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Improvement of regeneration of *Lycopersicon pennellii* protoplasts by decreasing ethylene production

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Summary. *Lycopersicon pennellii* shoots, cultured *in vitro* for more than a year (type I plants) produced few viable protoplasts in contrast to shoots cultured *in vitro* for less than five months (type II plants). Ethylene production of both plant types was compared. The low viability of plant type I protoplasts could be correlated with high ethylene production and an increased cell sap osmolality. The ethylene action inhibitor silver thiosulphate improved protoplast yield and viability, especially when using donor tissue, germinated and cultured on medium containing silver thiosulphate (type III plants). Moreover, the choice of cell wall degrading enzymes influenced protoplast viability, since ethylene release was significantly lower using Cellulase R 10 than Cellulysin. All improvements together resulted in an efficient protocol for the isolation and regeneration of *Lycopersicon pennellii* protoplasts.

Abbrevations: ACC: 1-Aminocyclopropane-1-carboxylic acid, FW: Fresh Weight, Mes: 2-Morpholino ethane sulphonic acid, NMU: N-Nitroso-N-Methyl-Urea, PE: Plating Efficiency = Number of calli / number of protoplasts x 100%, Pps: protoplasts, STS: Silver thiosulfate.

Introduction

Ethylene is a gaseous plant hormone which is produced by plant cells mainly as a response to stress conditions (Yang and Hoffman 1984). Several researchers have attempted to elucidate the possible influence of ethylene on plant tissue cultures (Burg 1962; Pratt and Goeschl 1969). It has been shown that the accumulation of ethylene in tightly closed culture vessels can have a dramatic effect on the morphology and physiology of the developing shoots (Hussey and Stacey 1981, 1984), resulting in plants with a stunted phenotype. Many authors have investigated the enzymatic induced release of ethylene during protoplast isolation from different plant sources (Mussell et al. 1986; Facciotti and Pilet 1981; Cassells et al. 1980, Cassells and Tamma 1986; Perl et al. 1988). The frequently observed low viability and regeneration capacities of protoplasts were related to high ethylene levels.

Inhibition of ethylene production in tissue culture procedures often improves the physiological state of the donor tissue and increases the viability of isolated protoplasts. The suppression of ethylene action by silver ions was first reported by Beyer (1976). Since then silver ions have successfully been applied to promote shoot regeneration in several plant taxa (Purnhauser et al. 1987; Songstad et al. 1988) and improve protoplast viability (Perl et al. 1988).

We are currently engaged with the induction of antibiotic resistances in tomato species, by treating protoplast populations with the mutagen NMU (Jansen et al. 1990). Therefore, a standardised procedure for the isolation of large protoplast populations with high viability and regeneration capacities is needed. For most of the used species within the Lycopersicon genus such a protocol is readily available. However, with the tomato species *Lycopersicon pennellii* we had difficulties obtaining sufficient protoplasts which would survive throughout our experiments. We observed that *Lycopersicon pennellii* plants that had been subcultured in vitro every 6 weeks for more than a year (Type I plants) produced protoplasts with poor regeneration capacities compared to protoplasts derived from plants that had been grown in vitro for less than five months (Type II plants). As stated earlier, ethylene production and accumulation could be an important factor to take into account. We tested the hypothesis whether there would be a difference in ethylene production between the two plant types and whether this difference could be correlated with the variation in regeneration capacity of protoplasts. Therefore, we altered several steps of the protoplast isolation protocol that would either prevent or decrease ethylene accumulation.

In this paper the ethylene production of shoots and protoplasts of plant type I and II is compared. In order to improve protoplast yield and regeneration we have examined effects of silver thiosulphate and silver nitrate,

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Table 1. Comparison of protoplast yield and plating efficiency of plant type I and II shoots cultured with or without STS.

donor tissue [a]	STS (mg/l)	pps yield (x 10^6 / g FW)	plating efficiency (%)
Type I	0	1.8	1
	2	3.1	4
	5	2.7	2
Type II	0	2.4	10
	2	3.6	18
	5	3.2	14

[a] Data represent the averages of nine determinations. Each experiment is performed with approximately 3 grams of tissue

inhibitors of ethylene action. Furthermore, we report on the induction of ethylene by cell wall degrading enzymes. All tests were performed with plant type I and II, to find an explanation for differences in protoplast viability observed between these two plant types.

Materials and methods

Plant material. Seeds of *Lycopersicon pennellii* (LA 716) were originally obtained from the Centre for Plant Breeding Research (CPO) Wageningen. Shoot segments with at least one node, derived from these seeds, were cultured in sterile glass containers on MS medium (Murashige and Skoog 1962), supplemented with 1.8% sucrose and 0.8% Difco agar, at 25° C, 2000 lux and a 16/8 photoperiod. Containers were closed with lids that contained a rubber valve for ethylene sampling.

Where indicated aqueous filter sterilized solutions of STS, $AgNO_3$ or ACC were each added to autoclaved medium at the required concentrations.

Protoplast isolation. Three to four week old shoots were used for protoplast isolation. Prior to isolation the shoots were transferred to 4 °C in the dark for 4 hours, according to the method of Tan et al. (1987). About 0.25 g of leaves from the top shoot were cut (feather shaped) and incubated for 1 hour in a preplasmolysing solution [CPW salts (Frearson et al. 1973), 7.5% mannitol, 3mM Mes, pH 5.8]. Subsequently the leaves were transferred to 9 cm petridishes containing an enzyme mixture dissolved in 10 ml CPW salt solution. For ethylene experiments leaves were incubated in 25 ml Erlenmeyer flasks sealed with airtight rubber valves. Incubation was for 16 hours in the dark at 25 °C. After enzyme incubation ethylene levels were determined. The leaves were then gently resuspended with an automatic pipet and the mixture was filtered through a 70 µm sieve. The filtrate was diluted with an equal volume of CPW salts and centrifuged for 5 min at 600 rpm. The pellet was resuspended in 6 ml CPW salts on top of 3 ml of a CPW 18% sucrose solution. Intact protoplasts were collected by floatation on this sucrose solution after centrifugation for 6 min at 800 rpm. To remove the sucrose the protoplasts were resuspended in W5 washing medium (Menczel and Wolfe 1984) and collected by centrifugation for 5 min at 600 rpm. A haemocytometer was used to determine the number of isolated protoplasts. Protoplasts were cultured according to the method of Jansen et al. (1990).

Ethylene measurements. 500 µl gas samples were taken using a gastight Hamilton syringe and ethylene levels were determined by gas chromatography, using a Hewlett Packard gas chromatograph, equipped with a Porapak R column (6' x 18' ss, 80/100 mesh). Injector and detector temperatures were 60 and 220 ^{0}C respectively.

Osmolality determination. Cell sap osmolality was determined according to the procedure of Smith et al. (1984).

Results and Discussion

The physiological condition of the donor tissue is of great importance in protoplast isolation procedures. Parameters, like leaf length and width, contribute to higher protoplast yields per gram fresh weight (Cassells and Cocker 1982). In our experiments reasonable yields (10^6 protoplasts/g FW) of protoplasts with good plating efficiencies (15-18 %) were only obtained using plant material that had been kept in culture for less than five months (type II). In contrast, somewhat lower yields of protoplasts with poor dividing properties were obtained from shoots that had been grown *in vitro* for more than a year (type I; Table 1).

Since ethylene production of plants, used for protoplast isolation, could influence protoplast viability, we followed ethylene production for both plant types during 4 weeks. Indeed, our experiments demonstrated that type I plants, producing protoplasts with poor regeneration capacities, accumulated more ethylene than type II plants during culture on MS medium, reaching a maximum after 21 days in culture when they are used for protoplast isolation (Fig 1).

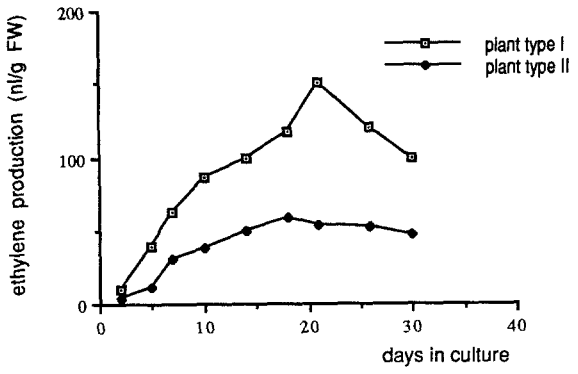

Fig 1. Ethylene production of type I and II plants during culture on MS medium for 30 days. Data are averages of two experiments.

Table 2-a. Comparison between enzymatically released protoplasts of plant type I and II in ethylene production, protoplast yield and plating efficiency .

	plant type I			plant type II		
enzymes used[a] (% w/v)	ethylene prod.[b] (nl / g FW)	pps yield ($x10^6$/gFW)	PE (%)	ethylene prod. (nl /g FW)	pps yield ($x10^6$/gFW)	PE (%)
0.9 cellulase	560	3.2	5	172	2.4	18
0.8 cellulase	910	2.3	2	402	2.5	16
0.7 cellulysin	1699	2.9	0	663	2.8	2
0.6 cellulysin	1306	1.8	0	620	2.6	4
0.9 cellulase +STS	214	2.6	7	–	–	–
0.6 cellulysin +STS	296	2.8	0	–	–	–
none	61	0	0	43	0	0

a) Macerozyme concentration used: 0.1 % w/v
b) Data represent the averages of at least four determinations

These results prompted a more detailed study on the effect of inhibitors of ethylene action. Silver thiosulphate STS) and silver nitrate ($AgNO_3$) were chosen as ethylene action inhibitors because of their successful application with other plant taxa (Beyer 1976; Veen 1985; Purnhauser et al. 1987; Perl et al. 1988). ACC, a precursor of ethylene, was used as control treatment on the effect of ethylene. Seeds were germinated on media containing $AgNO_3$, STS or ACC in different concentrations. Seedlings germinated on 5-40 mg/l ACC showed a drastic change in morphology as compared to the control treatment. Roots and cotyledons hardly developed and had a dwarfish appearance. (Fig 2a). Addition of $AgNO_3$ or STS to the medium resulted in a better development of roots and cotyledons (Fig 2b and c). For practical reasons we used STS in further investigations

When protoplasts were isolated from plant type I or II shoots and cultured on different concentrations of STS for three weeks, protoplast yield and plating efficiency improved and were optimal with STS in a concentration of 2 mg/l (Table 1). However, protoplast regeneration mainly increased where plant type II had served as donor tissue. These results indicate the necessity of using plant type II tissue in order to obtain protoplasts which can be regenerated efficiently.

Optimizing the condition of the donor tissue did improve protoplast yield and viability, but could still not be described as optimal. Therefore, we investigated another important step in the protoplast isolation protocol: the enzymatic digestion of the cell wall.

Cellulysin (Calbiochem), which we use as an enzyme for protoplast isolation, releases protoplasts with relatively low regeneration capacities from *L. pennellii* (Table 2-a). Since Cellulysin is a concentrated enzyme preparation (11000 units cellulase / g) we decided to use the enzyme Cellulase " Onozuka-R-10 ", which contains less purified cellulase (1300 units cellulase /g, Serva) in varying concentrations. This treatment resulted in a comparable amount of protoplasts isolated, however, with increased viabilities. Again, optimal results were obtained using plant type II tissue (Table 2-a). We investigated whether ethylene could be involved in the observed differences. Ethylene production during the

Table 2-b. Effect of three cell wall degrading enzymes in different concentrations on ethylene production, yield and plating efficiency of protoplasts, isolated from type III shoots.

Cellulase (% w/v)	Cellulysin (% w/v)	Macerozyme (% w/v)	Ethylene[a] (nl/g FW)	Yield pps (x 10^6 /g FW)	Plating efficiency %
.9	–	0.1	402	2.9	12
0.8	–	0.1	172	n.d[b].	17
0.8	–	0.08	210	2.9	15
0.7	–	0.1	404	2.8	13
0.7	–	0.08	238	2.3	15
0.7	–	0.07	235	n.d.	14
0.6	–	0.08	310	2.4	13
0.5	–	0.1	269	1.8	18
–	0.7	0.1	665	4.8	13
–	0.7	0.08	675	3.2	12
–	0.4	0.1	610	2.5	10
0	0	0	41	0	0

a) Data represent averages of at least three determinations. Each experiment is performed with approximately 3 gram tissue.
b) n.d. = not determined

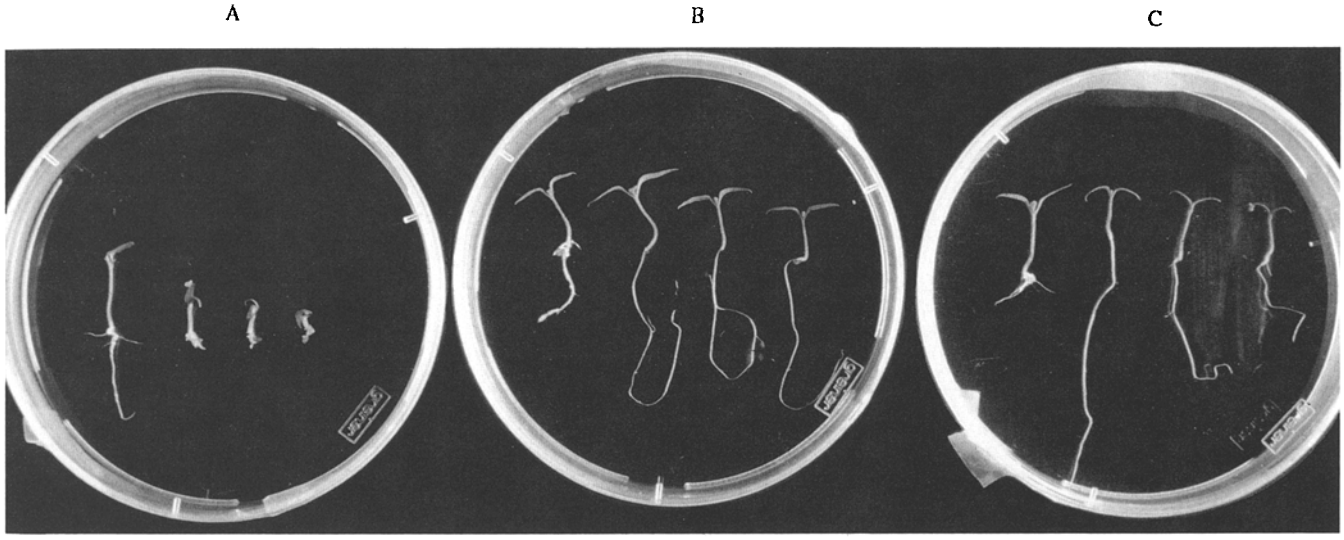

Fig 2. L. pennellii seedlings (8 days old), germinated on medium containing different concentrations of ACC, STS and $AgNO_3$. A: 0, 5, 20 and 40 mg ACC/l. B: 0, 4, 10 and 15 mg STS/l. C: 0, 5, 10 and 15 mg $AgNO_3$/l

enzymatic release of protoplasts from plant tissue is a frequently observed phenomenon and could be correlated with decreasing viability (Cassells et al. 1980; Facciotti and Pilet 1981; Anderson et al. 1982, 1985; Cassells and Cocker 1982). Ethylene production was monitored during enzymatic induced protoplast release from plant types I and II and compared with protoplast yield and plating efficiency. To ensure that most of the ethylene production during the release of protoplasts is induced by the cell wall degrading enzymes, we measured ethylene production of a control treatment (without addition of enzymes to the incubation medium). The results in Table 2-a demonstrate that most of the ethylene production is induced by the activity of the enzymes. Our results showed that plant type I protoplasts produced more ethylene compared to plant type II protoplasts when the same enzyme combinations were applied (Table 2-a). Protoplasts released by Cellulysin produced more ethylene than protoplasts released by Cellulase. This is not due to a difference in cellulase units between the enzymes, since applying them in equal amounts of units (0.11 % Cellulysin and 0.9 % Cellulase) still resulted in a higher ethylene production with cellulysin (1022 nl versus Cellulase 609 nl). Anderson et al. (1982, 1985) ascribed the high ethylene induction by cellulysin to a specific factor in this enzyme which induces ACC synthase. Higher concentrations of Cellulysin induce more ethylene, this is not the case when Cellulase is applied with plant type I or II (Table 2-a); a higher concentration of Cellulase induced less ethylene and subsequently gave rise to protoplasts with higher plating efficiency. Protoplast plating efficiency of both plant types differed significantly. Plant type I protoplasts, producing high amounts of ethylene, showed low regeneration capacities compared to the low ethylene producing protoplasts of plant type II.

A correlation between ethylene production and protoplast viability was obvious (compare Cassells et al. 1980, Facciotti and Pilet 1981). Addition of STS to the shoot culture medium of type I plants decreased ethylene accumulation of the isolated protoplasts (Table 2-a). However, this treatment did not significantly improve protoplast plating efficiency. The chronic exposure of the cells to ethylene has probably inflicted too many changes in the cells to be reversed with an STS treatment. Therefore we decided to continue further research using plants which had been germinated and cultured on medium containing STS, afterwards referred to as plant type III, in order to evade the problems which could arise from prolonged exposure to ethylene.

With plant type III tissue we further optimised the protoplast isolation and regeneration protocol by investigating the effect of various concentrations of cell wall degrading enzymes on ethylene production, yield and plating efficiency of protoplasts. We observed that plant type III protoplasts released by Cellulysin produced more ethylene than protoplasts released by Cellulase. However, ethylene levels were reduced in comparison to plant type I and II. A correlation between ethylene and plating efficiency was no longer visible probably because ethylene production of type III plants was reduced by STS (Table 2-b). Varying the Cellulase or Macerozyme concentrations did not significantly alter ethylene production. In general, compared to type I protoplasts, ethylene production by type III protoplasts was reduced and protoplast viability improved.

The observed differences between the plant types could be due to an alteration of the cell membrane of type I cells, possibly induced by:

1) environmental changes, which can lead to a different response to the routinely used cell wall degrading enzymes and subsequently in ethylene production.

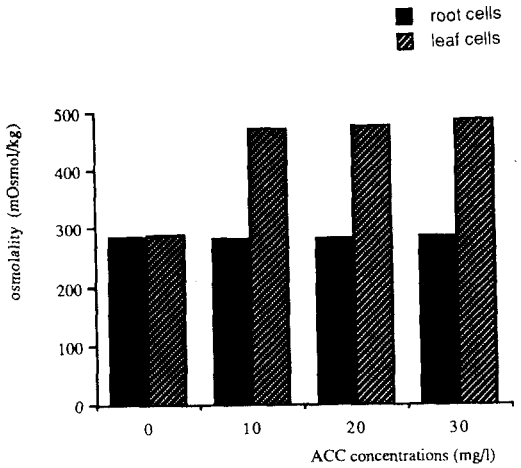

Fig 3 Leaf and root cell sap osmolalities of type II shoots, cultured for two weeks on medium containing 0-30 mg ACC/L.
(Data are averages of three experiments, s.d. is 20 mOsmol)

2) a repeated build up of ethylene in the culture vessels, which has irreversibly changed the plant cells, possibly the cell membranes. An indication, supporting the second possibility, is provided by the observation that ethylene can induce changes in the cell wall matrix of plant cells and can cause a reorientation of cellulose microfibrils (Beyer 1976; Mattoo and Lieberman 1977; Abeles 1985). This can result in an increase of cell sap osmolality as described by Eisinger et al. (1983).

Comparing cell sap osmolalities of plant type I and II leaves we discovered that type I plant cells, which have been exposed to ethylene repeatedly, showed a drastic increase in cell sap osmolality (388 mOsmol/kg) when compared to type II plant cells (291 mOsmol/kg) Culturing type II plants in medium containing 10 - 30 mg/l ACC also resulted in an increased osmolality of leaf cells (Fig 3). The results do support a role of ethylene in altering the cell sap osmolality. As expected, ethylene production of these shoots showed a positive correlation

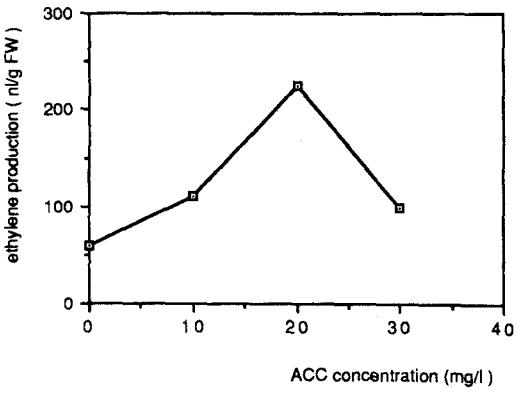

Fig 4. Ethylene production of type II shoots cultured on different ACC concentrations for two weeks.
(Data are averages of three experiments)

with ACC concentrations up to 20 mg/l (Fig 4). Addition of ACC to the culture medium in concentrations exceeding 30 mg/l induced yellowing of leaves, followed by leaf abscission. This could explain the decrease in ethylene production with 30 mg/l ACC shown in Fig 4. A remarkable observation was that the osmolality of root cells remained constant with increasing ACC concentrations (Fig 3).

Concluding we state that the lack of ethylene is an important component for improving plating efficiency of *L. pennellii* protoplasts. Differences in protoplast yield and -viability observed between plant type I and II can be correlated to ethylene induced effects. The choice of the right donor tissue (type III shoots, which have been continuously cultured on medium with STS) and the constitution and concentration of the enzyme solution that induced minimal ethylene production mainly contributed to the development of an optimized protopast isolation protocol for *Lycopersicon pennellii.* This protocol now results in yields of 2-3.10^6 protoplasts / g FW and a plating efficiency of 15-18 %.

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